Chapter 1

General Introduction

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Introduction

The intestinal epithelium forms a unique interface for interactions between the body and food components. It allows selective passage of nutrients, while offering protection against harmful substances. Under physiological conditions, the epithelium maintains a peaceful relationship with the extremely large community of commensal bacteria that live in the intestine and influence our health. [1] The intrinsically dynamic epithelium with its ability to respond to luminal stimuli, provides ample possibilities to modulate intestinal physiology by nutrition and microbiota.

Cholesterol is of vital importance for vertebrate cell membrane structure and function. [2] Metabolites of cholesterol, such as bile salts (BS), steroid hormones and oxysterols, fulfill important biological functions. [3] Hypercholesterolemia however represents a major risk factor for cardiovascular disease. [4,5] Cholesterol homeostasis is tightly regulated by its intestinal absorption, fecal excretion and *de novo* synthesis. [6] Classically, fecal cholesterol excretion was believed to be primarily driven by cholesterol secreted via the *hepatobiliary* pathway. [7] However, it has very recently become apparent that direct secretion of cholesterol from the blood compartment to the intestine, the process now adopted "*TransIntestinal Cholesterol Excretion*" (TICE), plays a major role in fecal cholesterol disposal. [6,8] Reduction of cholesterol absorption and induction of TICE represent attractive targets to facilitate cholesterol disposal. Ideally, inhibition of cholesterol absorption and/or induction of TICE would be facilitated by simple dietary intervention.

BS are important for absorption of cholesterol, fat and fat-soluble vitamins. On the other hand, elimination of excess cholesterol from the body is partly facilitated by breakdown of cholesterol to BS in the liver and their subsequent fecal excretion. [9]

In this theses we assessed, in a quantitative manner, intestinal function with respect to its capacity to digest and absorb lipids (dietary fats, cholesterol and BS), and its capacity to secrete cholesterol under varying intestinal conditions.

In this general introduction, first of all a short overview of BS homeostasis [1] and dietary lipid (dietary fat and cholesterol) absorption [2] will be provided. Subsequently, the introduction will cover cholesterol homeostasis including the transport of cholesterol through plasma [3], the characteristics, analytical possibilities and (pharmacological) inhibition of cholesterol synthesis [4] and absorption [5], and finally pathways of cholesterol excretion [6].

1. Bile salt homeostasis

Under physiological pH (6-8) biliary and intestinal bile acids are present in the form of sodium salts [10] and will therefore be referred to as BS. BS are amphiphatic molecules, which are produced by the liver from cholesterol. BS stimulate bile flow and biliary phospholipid (PL) secretion, regulate their own synthesis, and are indispensable for efficient lipid absorption (including dietary fats, cholesterol and fat-soluble vitamins A,D,E and K). Chenodeoxycholate (CDC) and cholate are the major primary (produced by the liver) BS. The rodent liver converts the majority of CDC to more hydrophilic α- and β-muricholic acid (α-/β-MC). [11] Intrinsically, rodents show a more hydrophilic BS pool compared with humans. [12]

Nearly all BS are conjugated by liver peroxisomes, mainly with taurine in rodents and glycine in humans. [12,13] The liver secretes these BS conjugates across the canalicular membrane into the bile canaliculi.
This canalicular secretion occurs against a high concentration gradient and is facilitated by adenosine-triphospate (ATP)-dependent transporters, the most important of which is the Bile Salt Export Pump (BSEP or ABCB11). [14,15] Via the bile canaliculi, BS are finally transported to the duodenum where they facilitate the solubilization of lipids.
BS can be reabsorbed passively, but are mainly reabsorbed actively by the Apical Sodium-dependent BS Transporter (ASBT) [16] in the terminal ileum (overall ~95%). At the basolateral enterocyte membrane, BS leave the cells via the organic solute transporter (OSTα/β). [17] They are transported back to the liver through the portal system and are mostly taken up via the Na^+-dependent Taurocholate Co-transporting Polypeptide (NTCP) at the basolateral hepatocyte membrane. [18] This process is called enterohepatic cycling (EHC). [9] Under physiological circumstances BS transport from hepatocyte into the bloodstream is negligible. However, this may rapidly change under cholestatic conditions, where BS are delivered to the blood via members of the Multi drug resistance Related Proteins (MRPs), such as MRP4/ABCC4. [19]
The BS that escape absorption in the terminal ileum enter the colon. *Primary* BS can be deconjugated and converted to *secondary* BS species by the intestinal microbiota. Bacteria can convert β-MC to hyodeoxycholate (HDC) [20,21] and ω-MC. In addition, they are able to convert CDC to lithocholate (LC, mainly in humans) [22] or ursodeoxycholate (UDC) [23], and cholate to deoxycholate (DC) [22]. A part of colonic BS is passively absorbed, while the remaining part is excreted with feces. Excretion of BS with feces actually represents an important pathway for cholesterol disposal. Under steady state conditions, the liver compensates for fecal BS loss by *de novo* BS synthesis.
BS regulate their own secretion and synthesis via an elaborate feedback pathway. [24] Hepatic BS activate the nuclear receptor (NR) farnesoid X receptor (FXR), which induces BS secretion via BSEP and reduces basolateral BS uptake via NTCP.
After uptake in the terminal ileum, BS activate intestinal FXR which activates OSTα/β-mediated export of BS. FXR also induces the short heterodimer partner (SHP), resulting in release of fibroblast growth factor (Fgf) 15 (mice) or FGF19 (humans) into the portal blood. [24] Fgf15/FGF19 activates the fibroblast growth factor receptor 4 (FGFR4) in the liver, which ultimately facilitates transcriptional inhibition of *cholesterol 7 alpha-hydroxylase (Cyp7a1*, encoding the rate-limiting enzyme for BS synthesis). [25] Both SHP and FXR lack a *Cyp7a1* DNA binding site. Liver FXR is activated primarily by primary BS (mainly CDC), which induces SHP. SHP can activate the liver-related-homolog-1 (LRH-1, rodents) or α-fetoprotein transcription factor (FTF, humans). LRH-1/ FTF can bind the bile acid response element II (BARE-II) in the *Cyp7a1* gene, inhibiting its transcription. SHP blocks the interaction between hepatocyte nuclear factor 4α (HNF4α) and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1α), which also inhibits *Cyp7a1* transcription via BARE-II. *Cyp7a1* transcription can in addition be inhibited via steroid hormones, inflammatory cytokines, insulin, growth factors and NRs that operate independently of FXR. [26] LC is a ligand for the pregnane X receptor (PXR), constitutive androstane receptor (CAR; indirectly) and vitamin D receptor (VDR).

PXR and VDR bind the BARE-I and block recruitment of PGC1α, inhibiting *Cyp7a1* transactivation by HNF4α. CAR binds the BARE-II and competes with HNF4α for several coactivators, including PGC1α. [26] On the other hand, when the liver X receptor (LXR) is activated by oxysterols, transcription of *Cyp7a1* is induced via the BARE-I in rodents (not in humans due to an alteration in the BARE-I sequence), resulting in production of BS from cholesterol. [24]

It is becoming more and more clear that BS are not just simple detergents necessary for lipid absorption, but are also involved in the regulation of glucose, lipid homeostasis as well as energy expenditure. [27]

Recent data show that removal of BS from the intestine with sequestrants can decrease plasma low density lipoprotein (LDL) levels and hyperglycemia in patients with type II diabetes. [28] The mechanisms by which the total pool size and/or profile of BS influence different physiological processes are only beginning to be understood.

2. Dietary lipid absorption

2.1 Dietary fat absorption

Lipid absorption in general involves emulsification, lipolysis, micellar solubilization, uptake by mucosal epithelium, re-esterification, chylomicron (CM) formation and lipoprotein metabolism. [29] This paragraph will focus on dietary fat, whereas specific aspects of cholesterol absorption will be discussed in paragraph 2.2. Triglycerides (also called triacylglycerols; TG) form the major lipid component in the human diet. Lipids are dispersed by chewing, which increases the surface area. In the stomach, partial hydrolysis by gastric and lingual lipase takes place (10-30% of TG) and diacylglycerol and free fatty acids (free FA) are released. [30,31] Pancreatic lipase facilitates further hydrolysis, producing monoacylglycerol and free FA. [32] PL (from diet, bile and sloughed intestinal epithelial cells) are hydrolyzed by phospholipase A_2, yielding lyso-PL and free FA. [33] The products of pancreatic lipolysis have limited solublility and need subsequent solubilization by BS and/or association with PL. [34,35]

Adjacent to the luminal surface of enterocytes, micellar dissociation is promoted by decreased pH (5.3-6.0) [36] in the so-called unstirred water layer [37], allowing for diffusion of FA across the cellular membrane. FA can also be taken up by transporters such as FA transporter protein 4 (FATP4) and FA translocase (FAT/CD36), but none of these transporters were shown to be critical for FA absorption. [38,39]

In the enterocyte, FA may bind to the intestinal FA binding protein (IFABP) and diffuse into the endoplasmatic reticulum. After activation acyl-CoAs are produced and re-esterified into TGs via several steps. [40] Microsomal TG transfer protein (MTP) assembles TG, PL and cholesterol together with apolipoprotein B48 (ApoB48) to form a CM particle or with ApoB100 (not in humans) to form a very low density lipoprotein (VLDL) particle, both of which are secreted to the interstitium via the basolateral membrane. [41] From the intestitium the particles enter lymphatic capillaries that drain into omental lymphatic channels and eventually reach the systemic circulation via the thoracic duct. [40]

2.2 Cholesterol absorption

Cholesterol present in the intestinal lumen derives from several sources, including diet, bile, intestinal secretion and desquamated epithelial cells. In humans consuming Western type diets, 300-500 mg dietary cholesterol enters the intestinal lumen per day, whereas the contribution of biliary cholesterol has been estimated to be approximately 800-1200 mg per day. [42] Cholesterol is a hydrophobic molecule, and it intestinal absorption is facilitated via similar steps as described for FA (emulsification, hydrolysis of dietary esterified cholesterol, micellar solubilization, and uptake within enterocytes). In healthy humans normally approximately 50% of intestinal cholesterol is absorbed. [43] Micellar solubilization of cholesterol is essential for cholesterol absorption. [44] The physical chemistry of biliary cholesterol may influence its absorption in the intestine. Whereas micellar biliary cholesterol is readily available for absorption, dietary cholesterol first has to be released from food oils or tissue membranes. Elegant isotope infusion studies in rats, however, showed that only on high cholesterol diet dietary cholesterol is relatively malabsorbed compared with biliary cholesterol.

On low and moderate cholesterol enriched diets micellized and non-micellized cholesterol appear in lymph in similar amounts. [45] Biliary cholesterol is unesterified, whereas dietary cholesterol is partly esterified. Dietary cholesterol esters (CE) thus must be hydrolyzed by pancreatic carboxyl ester lipase (CEL) before cholesterol can be transported into enterocytes. Howles et al. showed that dietary esterified cholesterol absorption was reduced by >60% in CEL-null mice, whereas absorption of free cholesterol was normal. [46] Considering the low daily supply of intestinal CE [47], hydrolysis of CE by CEL may not be critical for overall cholesterol absorption. CEL may however serve a function similar to phospholipase A_2, in providing sufficient hydrolysis of PL (mainly phosphatidylcholine (PC), which is required for adequate micelle formation. For example, in rats drained of bile and pancreatic juice, administration of CEL enhanced lymphatic recovery of cholesterol infused into the duodenum as a micellar, PC containing, solution. [48] Moreover, CEL itself may be absorbed by enterocytes via endocytosis. [49] and the ceramidase activity of CEL has been implicated in proper intracellular cholesterol trafficking and CM assembly. [50]

In addition to cholesterol, a typical Western diet contains structurally similar phytosterols (including plant sterols (~95%) and stanols (~5%)), in similar amounts as cholesterol, depending on the diet.

Plant sterols and stanols inhibit the absorption of cholesterol, however the mechanism(s) by which they do so remain a matter of discussion (see paragraph 5.3.2). Uptake of cholesterol and plant sterols and stanols is believed to be facilitated by the Niemann-Pick C1 Like 1 (NPC1L1) transporter [51], which is located in the brush border membrane of enterocytes in the proximal (jejunum), but not the distal (ileum) small intestine. Npc1l1 null mice showed 70% and 90% reduction in cholesterol and plant sterol/stanol absorption, respectively, compared with control mice. [52,53]

In addition, Npc1l1 null mice showed upregulation of intestinal and hepatic HMG-CoA synthase mRNA and intestinal cholesterol synthesis. [53] In the proximal small intestine (and apical hepatocyte membrane), NPC1L1 co-localizes with ABCG5 and ABCG8 (figure 1).

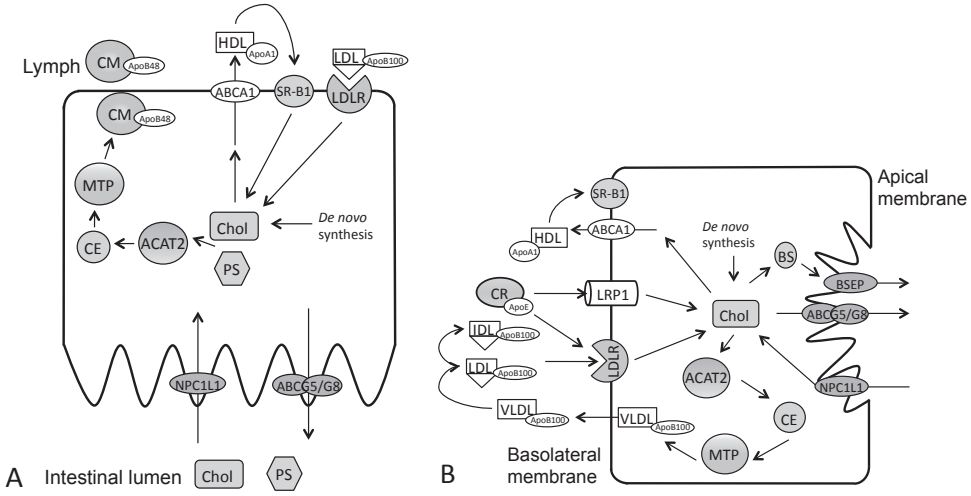

Figure 1. Cholesterol transporters, converting enzymes and lipoproteins in liver and intestine. **A**: Cholesterol in enterocytes originates from absorption, synthesis and uptake from the circulation. Cholesterol and plant sterols/ stanols are absorbed from the intestinal lumen via NPC1L1 and secreted back to the lumen via ABCG5/G8. Intracellular cholesterol and plant sterols/ stanols can be esterified by ACAT2, packaged into apoB48 containing CM by MTP, and secreted to the lymph. Alternatively, cholesterol can be secreted via ABCA1 in ApoA1 containing HDL particles. Uptake of cholesterol on the basolateral side occurs via SR-B1 (HDL-c) and LDLR (LDL-c), which recognizes ApoB100. **B:** The liver takes up HDL-c via SR-B1 and ApoE containing CR-c via LRP1 and the LDLR. In the hepatocyte ACAT2 esterifies cholesterol and CE are reassembled into VLDL particles together with CR-derived products. VLDL particles contain apoB100 instead of apoB48. ApoB100 binding to MTP results in loading of lipids to the VLDL particle.
The liver secretes the VLDL particles into the circulation for delivery of lipids to the periphery. VLDL is in part cleared by the hepatic LDLR, whereas the rest is transformed to IDL and LDL, which still contain apoB100 necessary for reuptake in the hepatocyte via the LDLR. The liver secretes cholesterol to bile via ABCG5/G8 and transforms cholesterol to BS, which are secreted via BSEP. Hepatic NPC1L1 can facilitate reuptake of biliary cholesterol.
Abbreviations: ACAT2: acyl CoA:cholesterol acyltransferase 2, CM: chylomicrons, CR: chylomicron remnants, LRP1: LDLR-related protein 1, MTP: microsomal triglyceride transfer protein, CE: cholesteryl esters, BS: bile salts, BSEP: bile salt export pump.

Efflux of unesterified cholesterol and plant sterols and stanols from the enterocyte back to the intestinal lumen, as well as biliary cholesterol secretion are facilitated by ABCG5/G8. [54,55] Intracellular cholesterol that is not effluxed by ABCG5/G8 travels to the endoplasmatic reticulum, where it is esterified by acyl CoA:cholesterol acyltransferase 2 (ACAT2). [56] When LXR is activated, it forms a heterodimer with the Retinoid X receptor (RXR) and the complex induces transcription of target genes encoding proteins involved in cholesterol disposal, including the two half-transporters ATP-binding cassette sub-family G member 5 and 8 (*Abcg5/ Abcg8*). [57] Transcription of *Npc1l1* on the other hand is downregulated upon LXR activation. This likely repesents an indirect effect, since LXR is not known as a direct repressor. [24]
Intracellular cholesterol is partly re-esterified and finally delivered to the bloodstream, mainly as component of CM, together with TG, PL and apoB48, for transport to the lymph. MTP regulates this process, and also facilitates VLDL formation after cholesterol esterification by ACAT2 in hepatocytes. [58]

In addition, enterocytic cholesterol can be transferred to apoAI high density lipoprotein (HDL) particles via ABCA1 (basolaterally located in enterocytes; figure 1). [59]

Up to now, only NPC1l1 seems to be critical for apical intestinal cholesterol import. [52,53] Studies, in which proposed alternative candidates, such as scavenger receptor class B member 1 (SR-B1), CD36 and caveolin-1, were eliminated, showed that none of these proteins was crucial for cholesterol uptake. [60-64] ABCA1 was implicated in cholesterol absorption in the past. However, unchanged fecal sterol excretion in Abca1$^{-/-}$ mice indicated that ABCA1 plays no role in control of cholesterol absorption. [65]

In vitro studies suggested a possible role for aminopeptidase N (CD13) in cholesterol absorption [66] which has not yet been confirmed in *in vivo* studies. In contrast to ABCA1, ACAT2 and MTP do affect cholesterol absorption (paragraph 5.3).

Cholesterol homeostasis

Disturbances in cholesterol homeostasis are associated with potential life-threatening consequences. Hypercholesterolemia promotes atherosclerosis and thereby represents a major risk factor for cardiovascular disease. [67,68] Pharmacological inhibition of cholesterol synthesis has been the most potent treatment option for hypercholesterolemia during the last decades. Cholesterol synthesis inhibition by itself however, can reduce the risk of cardiovascular disease by only a third. [69] This underlies the ongoing search for alternative therapeutic modalities.

The liver has been considered the major site of control in maintenance of cholesterol homeostasis. [70] The liver facilitates clearance of VLDL/ LDL particles and cholesterol-containing CM remnants, synthesizes cholesterol, synthesizes and secretes (nascent) HDL particles, secretes cholesterol and BS to bile and is involved in reverse cholesterol transport (RCT). [7]

RCT is classically defined as the process by which cholesterol from peripheral tissues is transported to the liver, followed by excretion via bile to feces in the form of neutral sterols and BS. In recent years, however, the importance of the intestine in many aspects of cholesterol physiology is increasingly recognized. The intestine has a major impact on cholesterol homeostasis at the level of cholesterol (re-) absorption, fecal excretion and *de novo* synthesis. [71] It has become apparent that, at least in mice, direct secretion of cholesterol from the blood compartment into the intestine, i.e. TICE, plays a major role in disposal of cholesterol via the feces. [72]

It would be desirable to induce fecal cholesterol loss via the intestine without increasing biliary cholesterol secretion and thereby the risk of cholesterol gallstones. [73]

3. Transport of cholesterol through plasma

A plethora of epidemiological studies have unequivocally shown that increased plasma cholesterol levels are associated with cardiovascular disease risk. Interestingly this does not necessarily coincide with increased tissue cholesterol, but is probably caused by changes in rates of secretion and uptake of cholesterol. [74] Cholesterol is a lipophilic molecule which is transported through blood in lipoproteins.

The type of lipoprotein is determined by its buoyant density and apoprotein composition, which act as emulsifying coating and target their metabolism. [75]

There are marked differences in lipoprotein metabolism between humans and rodents. For example, mice do not possess cholesterol-ester transport protein (CETP) and have an up to 40-fold higher LDL clearance by the liver compared to humans. [70,76] Mice carry most of their plasma cholesterol in HDL particles and as such are not a good model for human disease. [70,76]

This is the reason why many studies on cholesterol metabolism have been performed in mice with genetic deficiency of major determinators of plasma cholesterol metabolism such as the Ldl receptor (Ldlr) [77] or ApoE [78]. More recently "humanized" mice have become available in which human CETP [79] is expressed. On an Ldlr null or ApoE3 Leiden background these mice mimic many aspects of the hyperlipidemic human phenotype. [80,81] Inhibition of CETP increases HDL and should ameliorate atherosclerosis, but clinical trials with the first CETP inhibitor (torcetrapib) were terminated because of adverse off-target effects (increased mortality and cardiovascular events).

Recently, a new CETP-related drug (dalcetrapib) showed lack of a clinically meaningful benefit in a clinical trial, and further testing of the drug has been halted.[82] Until now, inhibition of cholesterol synthesis has remained the first line of therapy for hypercholesterolemia.

4. Cholesterol synthesis

4.1 Control of cholesterol synthesis

Cholesterol is synthesized from its precursor unit acetyl-CoA via a complex metabolic pathway summarized in figure 2. [83,84] HMG-CoA reductase is the rate-limiting enzyme in cholesterol synthesis [85]. Recently squalene monooxygenase, which catalyzes the first oxygenation step in cholesterol synthesis, was suggested to represent a possible second control point in cholesterol synthesis beyond HMG-CoA reductase. [86] Cholesterol and fat biosynthesis are under control of a family of transcription factors designated Sterol Regulatory Element Binding Proteins (SREBPs). Three isoforms of SREBP have been described, i.e., SREBP1a, SREBP1c and SREBP2. Genes encoding enzymes and tranporters involved in cholesterol absorption and efflux are under tight transcriptional control (reviewed recently by [57]).

Cholesterol and its biologically active metabolites act as ligands for NR that regulate gene expression. LXR is a major regulator of cholesterol metabolism. [57] When sterols are present in excess intracellularly, LXR-mediated activation of SREBP1c transcription leads to induction of oleic acid synthesis. Oleic acid is the preferred FA for the synthesis of cholesterol esters (CE), which can be stored intercellularly. LXR activation thus protects cells from accumulation of excess free cholesterol, which is toxic and can result in cell death.

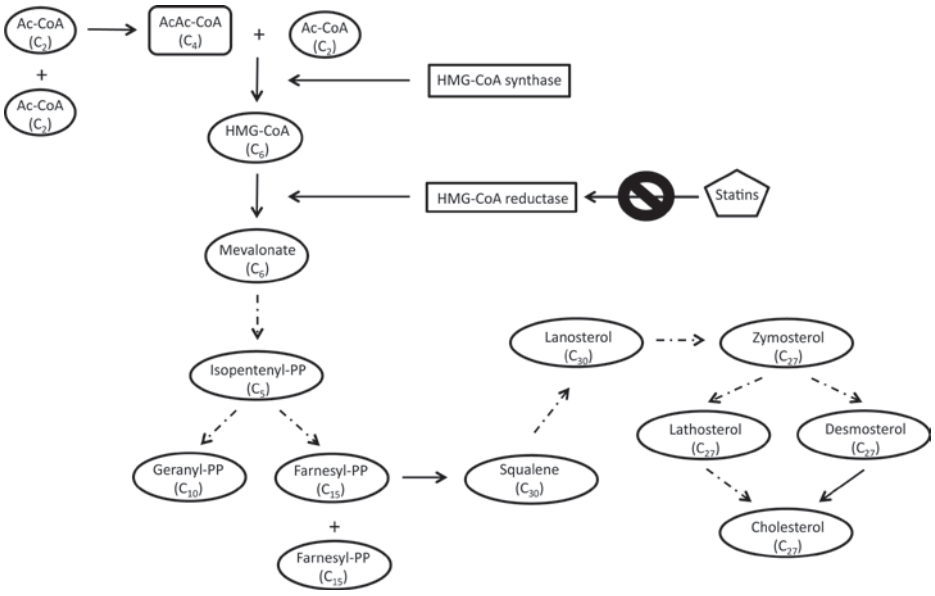

Figure 2. Cholesterol biosynthesis pathway. Cholesterol is synthesized from its precursor unit acetyl-CoA (Ac-CoA). Two acetyl-CoAs are condensed, forming acetoacetyl-CoA (AcAc-CoA). AcAc-CoA and a third acetyl-CoA are converted to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) by the action of HMG-CoA synthase. HMG-CoA is converted to mevalonate by HMG-CoA reductase. Mevalonate is subsequently converted to an isoprenoid molecule, isopentenyl pyrophosphate (PP), with the concomitant loss of CO_2. Geranyl-PP and farnesyl-PP are produced from isopentenyl-PP. Two farnesyl-PP subunits are combined to form squalene. Squalene is converted to lanosterol and subsequently cholesterol via many intermediates, including zymosterol, desmosterol and lathosterol. Solid line: direct step. Dashed line: product is formed via intermediate steps.

Cholesterol synthesis is tightly regulated by SREBPs (mainly type 2). When cellular cholesterol is high, SREBP2 is located to the ER in a complex with SREBP2 cleavage–activating protein (SCAP). When cells are depleted of sterols, SCAP escorts SREBP2 from the ER to the Golgi apparatus, where it is cleaved in order to release part of the protein from the membrane. SREBP2 then can enter the nucleus, bind to a sterol response element (SRE) in the enhancer/ promoter region of many target genes involved in cholesterol synthesis, and activate their transcription. [87] The importance of cholesterol synthesis for survival is illustrated by the fact that defects in the cholesterol synthesis pathway are generally lethal in mice. Complete loss of function of early cholesterogenic enzymes is rarely described in humans and deficiencies of these enzymes lead to severe malformations and disease.[88]

4.2 Measurement of cholesterol synthesis in vivo

The different methods available to determine cholesterol synthesis *in vivo* include sterol balance, (plasma) cholesterol precursor measurement and tracer incorporation techniques [such as deuterium incorporation (DI) and mass isotopomer distribution analysis (MIDA)], reviewed in ref. 89.

An early approach to estimate cholesterol synthesis has been cholesterol balance: measurement of cholesterol and BS excretion in feces followed by subtraction of dietary cholesterol intake yields whole body synthesis. Surrogate serum markers of cholesterol synthesis (and absorption) will be discussed in section 3. Here we will briefly discuss the two more recently developed and refined methods: DI and MIDA. The DI (^{2}H) method has a similar background as the tritiated (^{3}H) water method used in experimental animals. The theory behind the method is described below. Deuterated water can equilibrate in total body water and NADPH. Enrichment of deuterium in plasma water, representing the precursor pool, and deuterium enrichment of cholesterol in either plasma or red blood cells can be determined sensitively by isotope ratio mass spectrometry.

Eighteen acetyl CoA units containing 36 carbon (C) atoms are utilized to synthesize one molecule of cholesterol, which contains 27 C atoms and 46 hydrogen (H) atoms. During synthesis of a cholesterol molecule, 7 H atoms are incorporated originating directly from water, while another 15 H atoms are inserted from nicotinamide adenine dinucleotide phosphate-oxidase (NADPH).

H atoms from water may also become incorporated into substrates that, later on, are used for the generation of the cytosolic acetyl CoA pool that is used for cholesterol synthesis. If no labeled H from ^{2}H water were incorporated into these precursors of the cytosolic acetyl CoA pool, and if the reductive H of the NADPH were derived entirely from unlabeled sources, only 7 labeled H atoms (those orginating from the administered ^{2}H water) could be found in each newly synthesized cholesterol molecule (minimal theoretical labeled H/C incorporation ratio would be 7/27). Alternatively, if NADPH fully equilibrates with ^{2}H labeled water, then a final labeled H/C incorporation ratio of 22/27 would be found. This is considered the maximal labeled H/C ratio in short term measurements. [90] On the long run, an even greater value might be obtained when there is significant incorporation of labeled H into an important acetyl-CoA precursor, which by then has been used for cholesterol synthesis.

On a theoretical basis it is difficult to predict exactly how many labeled H atoms will be incorporated into each cholesterol molecule. Labeled H/C ratios are not available for all organs. In short term *in vivo* studies in mice and rats, 21-25 tritiated H atoms were incorporated into cholesterol molecules of whole carcass, liver or brain per C atom entering the biosynthetic pathway as acetyl CoA [90] and these values were subsequently used in other studies to calculate absolute cholesterol synthesis. However, combining the DI method with MIDA (discussed below) in long term experiments (up to 8 weeks), the maximum incorporation number in rat liver was found to be 30. [91] This could mean that earlier studies have overestimated true cholesterol synthesis rates. Many human studies using the DI method have used H incorporation values obtained from literature [92] and thus provide rough estimates of absolute cholesterol synthesis rates.

With MIDA the precursor-pool enrichment, fractional synthesis, and absolute (whole body) synthesis of cholesterol are calculated based on the pattern of excess enrichment among mass isotopomers of cholesterol present in plasma after administration of stable isotope-labeled ^{13}C-acetate. [93]

The distribution of abundances in newly formed cholesterol molecules, measured by gas-chromatography-mass spectrometry, allows calculation of the abundance in the precursor. MIDA thus eliminates the need to measure the precursor pool enrichment directly.

Knowing the true precursor-pool enrichment allows calculation of the fractional and absolute cholesterol synthesis rates. MIDA reveals the weighted mean abundance of the precursor pools contributing to the product mixture. [94]

In the past, human as well as animal studies applying MIDA were performed in relatively short time frames, which could underestimate whole body synthesis due to circadian effects and/or incomplete equilibration of the free cholesterol pool. A human study showed that the DI method and MIDA yield similar rates of fractional and absolute cholesterol synthesis when measured over at least 24h. [95]

4.3 The body cholesterol pools

A complication when assessing *de novo* cholesterol synthesis is the existence of different pools of cholesterol in the body with a differential rate of exchange. To incorporate this aspect, the turnover of plasma cholesterol has been modeled by dividing total body cholesterol into 2 or 3 exchangeable pools. [92,93]

In the 3-pool model, pool 1 (rapidly miscible) is considered to consist of cholesterol in fairly rapid equilibrium with plasma cholesterol (plasma, blood cells, liver and intestines), pool 2 consists of cholesterol that equilibrates at an intermediate rate (visceral and peripheral tissues) and pool 3 represents the slowest cholesterol turnover compartment (adipose and connective tissue, skeletal muscle and arterial walls).

The 3-pool model has been used for long term studies (up to 48 weeks). [92] Differentiation between pools 2 and 3 in short term experiments appeared not essential for accurate mathematical modeling. Pool 1 in the 2-pool model represents the rapidly exchangeable pool similar to that in the 3-pool model, whereas pool 2 of the 2-pool model represents a combination of pool 2 and 3 of the 3-pool model. [93]

The central nervous system contains a major part of total body cholesterol (15% and 23% in mice and humans, respectively). There is no detectable uptake of plasma cholesterol into the brain via the blood-brain-barrier. [96] The central nervous system presumably excretes only a very small amount of cholesterol to pool 3 under physiological conditions. Thus, it must be kept in mind that brain synthesis is not taken into account when calculating whole body cholesterol synthesis.

In general, whole body cholesterol synthesis rates measured using either the DI method or MIDA were shown to be comparable with those reported with the classical cholesterol balance (cholesterol intake + synthesis = fecal excretion of BS + neutral sterols). [93,97]

4.4 Regional and whole body cholesterol synthesis

Virtually every mammalian cell synthesizes cholesterol, in most animals the main part being synthesized in extrahepatic organs. [70,98,99] Whole body synthesis and the contribution of liver and intestine to whole body synthesis have been determined for several species.

A complication in evaluating the value of these measurements is the circadian rhythm of cholesterol synthesis, which has not always been taken into account (see below). It seems justified to conclude that species such as hamsters, guinea pigs, rabbits and squirrel monkeys and humans have much lower synthesis rates than rats and mice.

The gastrointestinal tract contributes around 15-35% to total cholesterol synthesis in experimental animals. In rodents, the contribution of the liver to whole body synthesis at night has been shown to vary from as low as ~15-20% in rabbits and guinea pigs to as high as 50% in rats. [70] In humans the liver is thought to contribute only around 10% to whole body synthesis [70] (~10 mg/kg/d). Low rates of local synthesis relative to the rates of uptake of newly synthesized cholesterol from blood in rats were found in adrenal glands, spleen, lung and kidneys. These organs increase their cholesterol synthesis when circulating levels of plasma cholesterol are decreased. [99]

4.5 Influence of diet and time of day on cholesterol synthesis

Whole body as well as organ specific cholesterol synthesis rates vary depending on the presence of cholesterol and other lipids in the diet. Dietary cholesterol suppresses hepatic cholesterol synthesis. In rats fed no dietary fat, liver synthesis rates are decreased when feeding a 2% cholesterol containing diet, while synthesis rates in the intestine and other extrahepatic tissue remain similar, suggesting that only the liver senses the increase in cholesterol uptake. [100] Adding cholesterol to either a low or high fat diet also leads to decreased hepatic cholesterol synthesis in hamsters. [101]

It appears that dietary fat alone however does not affect hepatic and whole body cholesterol synthesis, but can induce intestinal cholesterol synthesis. This was illustrated by several studies. First of all, on a high fat compared with low fat diet, hepatic cholesterol synthesis is comparable in hamsters. [101] In addition, it was shown in mice on a 0.2% cholesterol diet that dietary short, medium and long chain fatty acids did not differentially affect extrahepatic cholesterol synthesis. [102] Infusion of corn oil in non-cholesterol, non-fat fed rats increases cholesterol synthesis in the intestine, while liver synthesis remains the same. [100] A high fat diet in hamsters also increases cholesterol synthesis in the intestine in the presence of dietary cholesterol, however whole body synthesis is not affected [103].

In addition to dietary fat and cholesterol content, the type of diet influences cholesterol synthesis rates. These were shown to be lower on purified diets as compared to non-purified diets. [104,105] Energy restriction per se seems to have the greatest (lowering) effect on cholesterol synthesis (reviewed by [89]).

Both animals and humans display a circadian rhythm of cholesterol synthesis, with a peak in synthesis several hours after feeding. Most studies on cholesterol turnover in experimental animals have been performed during the dark phase of the light cycle. [106] It must be kept in mind that cholesterol synthesis is 2-3 fold higher during the (end of the) dark phase compared to the light phase. [93,107,108] In extrahepatic tissues the difference between day and night in cholesterol synthesis is much smaller compared with the liver, at least in mice. [107] Circadian rhythm in the liver is controlled by different molecular mechanisms, including cAMP-dependent and CLOCK/ BMAL1 regulation, which for example regulate expression of cholesterol synthesis genes *Hmgcr*, *Hmgcs*, *Sqs*, *Fpps* and *Cyp51*. For details, see ref. 109-111.

4.6 Targeting cholesterol synthesis in hypercholesterolemia

HMG-CoA reductase inhibitors (statins) have been used successfully to inhibit cholesterol synthesis and reduce all-cause mortality since the late 1980's. [112] Reduced hepatic cholesterol is compensated for by synthesis of LDLR to draw cholesterol out of the circulation [113] leading to lowering of plasma cholesterol levels. Some statins (pravastatin) seem to preferentially inhibit synthesis in liver and intestine (90%) [114,115] compared with synthesis in extrahepatic organs, such as kidneys (73%), testes (55%), lungs (53%), spleen (53%) and adrenals (49%). [114] Other statins (lovastatin) exhibit widespread cholesterol synthesis inhibition. [116] This may be mainly due to differences in cellular uptake [115], which can be caused by differences in lipophilicity, pH-sensitivity, plasma protein binding, activity of metabolites and transporter facilitated uptake and export. For example, simvastatin and lovastatin are administered as very lipophilic (tissue penetrating) lactone prodrugs, whereas other statins are administered in their active form. [117]

Different statins have different affinities for import proteins of hepatocytes such as organic anion transporting polypeptides (OATP) and NTCP. [118-121] In addition, polymorphisms in these transporters differentially affect statin uptake and (*in vitro*) affinity for transporters can differ between human and experimental animal cells. [121]

Although highly effective, statins do not produce the desired health effect in a significant group of patients and can cause severe side effects such as myalgia and myopathies (rhabdomyolysis), [122] in particular in case of polypharmacy. Statins decrease farnesyl and geranyl pyrophosphate (isoprenoids) and dolichol synthesis, reducing essential post-translational prenylation and N-linked glycosylation of proteins. This may result in myocyte and hepatocyte cell damage and even cell death. [123,124] Newer statins, such as the recently approved pitavastatin, with a distinctive metabolic profile, may display a reduced incidence of these adverse effects. [125] In addition, efforts are ongoing to produce new drugs targeting cholesterol synthesis at different levels of the pathway. Squalene synthase inhibitors for example increase farnesyl diphosphate while decreasing cholesterol synthesis. They are able to prevent statin induced changes in protein farnesylation and cell death *in vitro*. [124] Since these inhibitors do not target true rate-limiting steps in cholesterol synthesis, they may not effectively reduce cholesterol synthesis as a monotherapy in tolerable dosages.

Variations in efficacy of statins can be caused by many factors, including race (genetics), body weight and diet. Humans that inherently have lower cholesterol synthesis rates may respond less adequate to statin treatment. LDL-cholesterol (LDL-c) response to statins is lower in black compared to white people, which is associated with lower baseline LDL-c and variant haplotypes of the HMG-CoA reductase gene in black people. [126] Statins may induce expression of proprotein convertase subtilisin-like kexin type 9 (PCSK9) and SREBP2. PCSK9 is a circulating protein that impairs LDL clearance by promoting LDLR degradation. PCSK9 thus could diminish statin efficacy, hence PCSK9 inhibitors are currently tested in clinical trials. [127]

In general, cholesterol synthesis inhibition leads to a reciprocal increase in cholesterol absorption, [128] which has led to elaborate research to develop cholesterol absorption inhibitors (discussed below). With growing knowledge on the determinants of response to cholesterol lowering therapy, in the future it may become feasible to individually tailor treatment.

5. Measurement and inhibition of cholesterol absorption

5.1 Measurement of cholesterol absorption in vivo

The available methods to measure cholesterol absorption in humans and mice include (radiolabeled or stable) isotope based methods (fecal and plasma dual isotope method), intestinal perfusion and blood levels of surrogate markers such as plant sterols and intermediates of cholesterol synthesis. Details can be found in several reviews. [129,130] The intestinal perfusion method is the only method that can directly quantitate absorption of intestinal cholesterol in humans. [131,132] Widespread use of this method is limited by the need for intubation and radiation exposure.

The dual isotope ratio method has been optimized using stable instead of radioactive isotopes both in the plasma (ratio of intravenously and orally administered labeled cholesterol) [133] and fecal (ratio of orally administered labeled cholesterol and sitostanol in feces) dual isotope ratio method. [134] The fecal dual isotope ratio method is limited by requirement of 72h feces collection. The plasma dual isotope ratio method has been limited by measurement of single time point absorption, however this method can nowadays be applied for longer periods, at least in mice. [135]

The only approach available to easily estimate (relative) changes in cholesterol absorption in large scale studies is to measure plasma surrogate markers. These include the ratio of plant sterols (campesterol and sitosterol) or cholestanol (cholesterol metabolite) to cholesterol in plasma. [136] Surrogate makers have shown to be valuable to answer questions related to changes in cholesterol absorption during treatments. However, dietary factors (fat, cholesterol, plant sterols and stanols [137], drugs (statins) [138] and metabolic diseases (diabetes mellitus) [139,140] can disturb correct interpretation of surrogate markers levels. For example, the reduction in plasma LDL-c correlates poorly with baseline levels of noncholesterol sterol markers of absorption (campesterol) and synthesis (lathosterol) in African- and European-American men treated with ezetimibe (a cholesterol absorption inhibitor, discussed in paragraph 3.3.1), simvastatin or a combination of the two drugs. [141] Similarly, although ezetimibe + simvastatin treatment significantly reduces campesterol and sitosterol levels in patients with familial hypercholesterolemia, baseline cholesterol absorption status does not determine LDL-c lowering response to ezetimibe + simvastatin therapy. [142] It is currently recommended to use several rather than one serum marker, if possible in addition to absolute measurements. [143]

5.2 Targeting cholesterol absorption in hypercholesterolemia

Statin therapy is not sufficient to prevent cardiovascular disease risk in a substantial proportion of individuals. [144] To increase treatment efficacy considerable interest has arisen in dietary and pharmacological interventions that inhibit cholesterol absorption, possibly for combining this with inhibition of cholesterol synthesis . Most agents are nonspecific and require consumption in high daily quantities while modestly lowering plasma LDL-c. ACAT2 deficiency resulted in cholesterol malabsorption in mice, however only in cholesterol fed and not in chow fed mice. [145] MTP inhibition normalized plasma lipoprotein levels in a rabbit model for human homozygous familial hypercholesterolemia. [146]

However, this process is related to disturbed CM formation, not specific for absorption of cholesterol and not intestine specific as it induces fat accummulation in the liver. [146] We will not discuss inhibition of these intracellular factors. Ezetimibe represents an exception, as it relatively specifically decreases cholesterol absorption. The impact of dietary plant sterols and stanols is discussed since it has become apparent very recently that they might have an unexpected intestinal effect (see section 6). Other cholesterol lowering food components are discussed elsewhere [147]

5.2.1 Ezetimibe

Specific inhibition of cholesterol absorption, either from biliary or dietary origin, is accomplished after binding of ezetimibe to NPC1L1. [148] The compound is first glucuronidated in the intestine before travelling to the liver via the portal vein. Glucuronidated ezetimibe blocks the internalization of the NPC1l1/ cholesterol complex [149] but does not block cholesterol absorption completely.

In the Npc1l1 null mouse there is residual cholesterol absorption, indicating that another pathway of cholesterol absorption must exist. Ezetimibe does not inhibit this residual cholesterol absorption in Npc1l1 null mice, indicating that this residual pathway is ezetimibe independent. [52]

Studies in experimental animals indicated that ezetimibe may decrease absorption of vitamin E (α-tocopherol), but not vitamin A and D. [150,151] However, ezetimibe did not seem to affect fat soluble vitamin status in patients. [152,153] Ezetimibe can decrease plasma TG concentration. This does not seem to be absorption dependent, but rather a secondary effect of ezetimibe induced hepatic LDLR expression and subsequent increased clearance of ApoB100 and ApoB48 containing lipoproteins, particularly during co-treatment with statins. [154]

In humans, ezetimibe alone decreases cholesterol absorption by 54% and decreases plasma LDL-c by ~19%, but increases whole body synthesis by 89%. [43] Combined treatment with statins for hypercholesterolemia, should therefore be very effective. [155] Recently however, the effect of combination therapy has become controversial. Some trials reported no additive effect of ezetimibe or even a worse atherosclerosis outcome when ezetimibe is added to patients previously treated with statins. [156] Others report increased efficacy of combination therapy over statin monotherapy, however data are limited to the level of serum lipid profiles. [157-160] Due to the lack of well-executed long term trials it is presently unclear whether combination therapy is better than increasing statin dosage. In the future, ezetimibe may be prescribed in patients in whom statin therapy is inadequate or intolerated. As monotherapy ezetimibe may improve postprandial hyperlipidemia and endothelial dysfunction. [161] In the presence of pancreatic dysfunction, CEL inhibitors may provide additional inhibition of cholesterol absorption when co-administered with ezetimibe. [150]

5.2.2 Plant sterol/ stanol supplementation

In contrast to cholesterol, plant sterols and stanols can not be synthesized by the body and are poorly absorbed (plant sterols 0.4-3.5% and plant stanols 0.02-0.3%). [162] Fractional absorption of different plant sterols and stanols varies depending on their side chain length. [163]

The role of ABCG5/G8 in intestinal and hepatobiliary efflux of plant sterols and stanols versus cholesterol is not completely clear. *In vitro* studies showed comparable effectiveness of plant sterol and cholesterol transport via ABCG5/G8. [164] However, loss of ABCG5/G8 function in mice results in hyperabsorption of plant sterols and stanols, but not cholesterol, implying that ABCG5/G8 is more important for efflux of plant sterols and stanols compared to cholesterol. [165] In contrast to cholesterol, plant sterols and stanols are poor substrates for ACAT2 [166], which seems to be the major factor limiting their absorption. Plant sterols and stanols effectively lower plasma LDL-c levels in adults as well as children with (familial) hypercholesterolemia. [167]

Plant sterols and stanols naturally occur in foods such as oils, (wheat) cereals, nuts and seeds. There are large differences in plant sterol/stanol consumption throughout the world, within the Western population and particularly between vegetarians and meat consumers. [168] Plant sterols/stanols are added to food products such as margarine, yoghurt and juices. To enable emulsification, added plant sterols/tanols are often esterified and require hydrolysis before being able to compete with cholesterol for absorption. It was recently suggested that the hydrolysis products of esterified plant sterols/ stanols (stigmasterol and especially saturated stearic acid) together may be more effective at lowering cholesterol micellar solubility than free plant sterols/stanols alone. [169]

The mechanism via which plant sterols and stanols lower plasma cholesterol does however not seem to be confined to decreasing cholesterol solubilization in the intestinal lumen. A recent meta-analysis showed that despite differences in absorption, plant sterols and plant stanols have similar effects on plasma lipid profiles. [170] Smaller repeated doses (3 times 0.6 g/d) of plant sterol/ stanol margarine as well as a single high daily dose (1.8 g/d) are equally effective at lowering cholesterol absorption and plasma LDL-c. [171]

Given that plant sterols and stanols are taken up in enterocytes, it was hypothesized that cholesterol absorption may be decreased by plant sterols/ stanols via increased cholesterol secretion to the intestinal lumen, for example via ABCG5/G8 under transcriptional control of the Liver X Receptor (LXR). However, Abcg5/g8 and Lxr knock-out mice did not reveal any change in cholesterol absorption [172-174], implying that others factors are involved.

The combination of plant sterols with ezetimibe did not show an additive effect on (surrogate markers of) cholesterol absorption and plasma LDL-c in mildly hypercholesterolemic subjects in an open-label study which was not controlled for dietary plant sterol and stanol content. [175] A recent randomized, double-blind, placebo-controlled, triple cross-over study on the other hand showed that plant sterols and stanols further reduced cholesterol absorption (fecal dual isotope method) and further increased fecal sterol excretion compared with ezetimibe alone in mildly hypercholesterolemic subjects [176,177]. This study showed that the combination of statin and plant sterols and stanols exerted a minor additional effect on plasma LDL-c levels. Further studies in severe hypercholesterolemic patients may provide additional information on the usefulness of plant sterol/ stanol-ezetimibe combination therapy.

An adverse effect of plant sterol and stanol consumption could be disturbed micellar incorporation and absorption of fat-soluble vitamins.[178,179] The long term potential adverse effects of plant sterols and stanols, such as increased atherogenesis and tissue (liver, brain) accumulation of plant sterols and stanols may seem negligible, but await further study. [168,180]

6. Cholesterol excretion

6.1 Classical Reverse Cholesterol Transport (RCT)

The body can dispose itself from cholesterol predominantly in two ways: as neutral sterols (cholesterol and its intestinal bacterial degradation metabolites) or as acidic sterols (BS). Cholesterol and BS are excreted mostly with feces and only minimal amounts of cholesterol are additionally lost via the skin. The classic view of RCT includes the flux of cholesterol from peripheral tissues to the liver mediated mainly by HDL particles, and the subsequent secretion of this cholesterol by the liver in bile that is transported to the intestinal lumen, leading to fecal excretion of cholesterol.

Hepatobiliary RCT for decades has been considered the main, if not the only, pathway for cholesterol elimination. We will briefly discuss this pathway and then turn to other, more recently identified pathway involved in cholesterol excretion.The liver plays an important role in cholesterol homeostasis by regulating uptake of lipoproteins, cholesterol synthesis and cholesterol secretion (figure 1). Uptake of cholesterol is facilitated primarily via basolateral LDLR (LDL and VLDL uptake) and SR-B1 (HDL uptake). Cholesterol is basolaterally secreted from hepatocytes to the plasma compartment in VLDL and HDL particles and apically from hepatocytes to bile either directly as free cholesterol (via ABCG5/G8) or after conversion to BS (via the BS export pump (BSEP or ABCB11) [181] Biliary BS secretion is the main driving force for secretion of cholesterol and PL. However, studies in mice in which the PL transporter multi-drug resistance P-glycoprotein (Mdr2 or Abcb4) was eliminated [182], showed that PL secretion itself is also required for cholesterol secretion.

Cholesterol as well as plant sterols and stanols are secreted into bile mainly via ABCG5/G8.

Recent studies of mice with altered hepatic expression of Niemann-Pick C2 (NPC2, a cholesterol-binding protein that is involved in intracellular cholesterol trafficking in hepatocytes, but can also be secreted to bile [183]) revealed that NPC2 may positively regulate the biliary secretion of cholesterol, which was supported by the correlation between levels of NPC2 protein and cholesterol in human bile. Secreted NPC2 appears to specifically stimulate ABCG5/G8-mediated cholesterol efflux. It was suggested that NPC2 binds cholesterol and thereby accelerates the transfer of cholesterol to micelles via ABCG5/G8. [183]

In recent years it has become clear that the definition of RCT needs revision. [184] Elimination of cholesterol via feces, at least in mice, mainly occurs *not* via the classical hepatobiliary route, but via an alternative pathway, which is adopted Trans Intestinal Cholesterol Excretion (TICE).

6.2 Transintestinal Cholesterol Excretion

6.2.1 The concept of TICE

The TICE concept has been reviewed recently. [8,73,184,185] Here we will briefly describe the pathway and latest insights in its dynamics. It was shown in several mouse models with extremely low biliary cholesterol secretion rates (inactivation of Abcg5, Abcg8 or Abcg5/g8 and Mdr2 or overexpression of hepatic Npc1l1, see figure 3), that fecal neutral sterol excretion was unchanged or even increased [186-190]. This could not be explained by increased fecal loss of newly synthesized intestinal cholesterol. [191] At a stable dietary cholesterol intake, this suggested that cholesterol could be excreted directly from blood to feces via the intestinal mucosa.

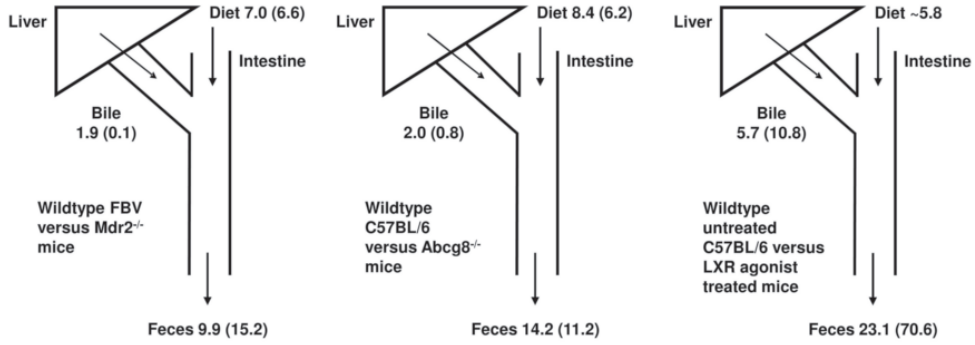

Figure 3. Schematic presentation of cholesterol input and excretion in several mouse models. Mice deficient in Abcg8 or Mdr2 have extremely low biliary cholesterol secretion rates, however fecal neutral sterol excretion is relatively similar (Abcg8$^{-/-}$) or even increased (Mdr2$^{-/-}$). Similarly, in wildtype mice treated with an LXR-agonist the sum of dietary and biliary cholesterol secretion is outleveled by fecal cholesterol excretion. These models provided the first evidence for the existence of transintestinal cholesterol excretion in mice.

Definitive indications for the existence of TICE were obtained in intestinal perfusion studies in which the bile duct was ligated and the absence of biliary components was compensated for by infusion of several (cholesterol free) model bile solutions. Van der Velde et al. demonstrated that TICE occurs in the entire small intestine, but particularly in the proximal part and plays quantitatively a more prominent role than the hepatobiliary route in mice. Importantly, intestinal cell shedding and synthesis could not account for the secreted cholesterol. [187,191] Brown et al. showed that mice with a targeted deletion of hepatic ACAT2 have normal biliary cholesterol secretion rates, in the presence of doubled fecal cholesterol excretion. The authors determined the fate of newly secreted liver-derived cholesterol. For this purpose, hepatic sterol pools were radiolabeled and nascent hepatic cholesterol-labeled lipoproteins were collected by isolated liver perfusion. The liver-derived lipoproteins were then re-injected intravenously into hepatic ACAT2 deficient and control mice to examine the movement of liver-derived radiolabeled cholesterol. It was found that radiolabeled cholesterol from perfusate of hepatic ACAT2 deficient mice was preferentially delivered to the proximal small intestine of wild type mice. [192]

A similar phenomenon probably occurs in mice deficient in the rate-limiting enzyme for BS synthesis (cholesterol 7α-hydroxylase, Cyp7a1). Cyp7a1 null mice have reduced BS synthesis and excretion and a smaller intestinal BS pool, which is associated with a major impairment in cholesterol absorption. Compared to wildtype mice, they have the same dietary intake and biliary cholesterol level. Nevertheless, fecal neutral sterol excretion greatly exceeds the sum of total dietary and estimated biliary cholesterol input to the intestine in these mice. [193]

Our lab contributed to the evidence by quantification of *in vivo* cholesterol fluxes from plasma and bile to feces by stable isotope methodology. [191] In these experiments, differentially stable isotope labeled cholesterol was administered to mice intravenously and orally to determine cholesterol absorption and label distribution over time in blood, bile and feces. In addition, cholesterol synthesis was measured by MIDA of blood, bile and fecal samples collected over time during administration of stable isotope labeled cholesterol precursor in drinking water. [191]

6.2.2 Transport of cholesterol from blood to feces

The transport of cholesterol from the blood compartment to the enterocyte and the subsequent excretion of cholesterol is probably facilitated by transport proteins. Sr-b1, being expressed on both the apical and basolateral side of enterocytes, was considered a possible candidate. However TICE was shown to be upregulated, rather than downregulated, in Sr-b1 knockout mice. [194] TICE was not altered by the absence of either Npc1l1 or Ldlr. [192,195] The relation between TICE and cholesterol efflux via Abcg5/g8 is somewhat puzzling [135,185] Compared with wildtype mice, TICE was decreased in Abcg5 knockout mice during LXR agonist treatment and plant sterol/stanol consumption. [135,191]. Strikingly however, TICE was not decreased by deletion of Abcg8 in mice. [187]

By gene expression analyses, so far no apical or basolateral transporters have been identified that regulate TICE. [8] At present it is unclear which lipoproteins are involved in donating the cholesterol to the TICE pathway and how it is targeted to the enterocytes. TICE does not seem to be mediated by HDL particles, based on unchanged fecal cholesterol excretion in mice deficient in HDL (Abca1 knockout mice). [65] Some evidence points to VLDL as a possible candidate origin for cholesterol involved in TICE. [192] Mice with liver specific depletion of Acat2 showed normal HDL levels, but increased delivery of liver-derived cholesterol to the lumen of the proximal small intestine as well as increased fecal cholesterol excretion. [65,192] Alternatively, TICE may not be facilitated via lipoprotein transport, but for example via increased delivery of erythrocyte cholesterol to the intestinal lumen. Additional research is required to identify key players in the pathway.

6.2.3 Stimulation of TICE

It is currently unclear whether or not ezetimibe, via inhibition of cholesterol absorption, could stimulate TICE. One study suggested pharmacological activation of TICE with ezetimibe. [196] However, this was not confirmed in intestinal perfusion studies [195] indicating that several pathways may co-exist in TICE.

TICE can be stimulated with pharmaceuticals such as nuclear receptor agonists of LXR [189,191] and peroxisome proliferator-activated receptor delta (PPARδ). [195] A high fat (low cholesterol) diet also induces TICE. [194,197]

Dietary plant sterols were shown to inhibit cholesterol absorption in mice. Brufau et al. recently found that dietary plant sterols/ stanols dose dependently induce fecal neutral sterol excretion, without affecting biliary cholesterol secretion. These data provide new insight into the cholesterol lowering mechanism of action of plant sterols/ stanols. [135]

A recent study showed that mice with acute biliary diversion are able to secrete labeled cholesterol derived from intraperitoneally injected cholesterol-loaded macrophages to the intestinal lumen. [198]

This study adds to the evidence that TICE may be quantitatively more important than hepatobiliary cholesterol secretion in the elimination of cholesterol via fecal excretion (RCT).

6.2.4 TICE in humans?

Although the available evidence suggests that TICE may be present in humans [199,200], it is at present unclear if TICE is present under healthy conditions and whether it can be stimulated pharmacologically or, preferentially, by dietary means. It has been estimated that TICE may contribute to one-third of fecal excretion in humans. [187] The possibility of TICE has never been tested in models for predicting plasma cholesterol. In the future measurement of body cholesterol kinetics by stable isotope studies in humans may provide new insight into the excretion and regulation of plasma cholesterol, and the dietary and therapeutic strategies to decrease hypercholesterolemia and associated cardiovascular disease in man.

Scope of this thesis

The specific aim of the research described in this thesis was to determine the effect of manipulation of intestinal function (by dietary, pharmacological and genetic intervention) on lipid homeostasis, in particular cholesterol excretion. We hypothesized that acceleration of intestinal transit time could induce fecal lipid excretion. This could be desirable in case of hypercholesterolemia. On the other hand, many children nowadays suffer from constipation and are treated with oral laxatives. Administration of these laxatives could negatively impact on their intestinal absorbtive function and lead to fecal lipid loss. In **chapter 2**, we describe the effect of acceleration of intestinal transit on absorption of dietary fat and cholesterol. In **chapter 3**, we studied the effect of accelerated intestinal transit on BS homeostasis and intestinal microbiota. Changes in the composition of dietary fat have been shown to alter fecal fat excretion. For example, lowering the ratio of polyunsaturated to saturated fatty acids (P/S ratio) was shown to induce fecal fat excretion.In **chapter 4**, we studied the effect of two high fat diets with different P/S ratios on cholesterol homeostasis. We used stable isotope methodologies to determine cholesterol absorption, synthesis and we modified the method previously used [191] to determine the origin of fecal sterols. Bsep$^{-/-}$ mice were previously shown to display defective biliary BS secretion. However, this was by far not as prominent as in their human couterparts. [15] In **chapter 5**, we studied the effect of absence of hepatic Bsep in mice on cholesterol homeostasis.

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